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INFLUENCE OF PREHEATING, pH, AND HOLDING TEMPERATURE UPON VIABILITY OF BACTERIAL SPORES STORED FOR LONG PERIODS IN BUFFER SUBSTRATES

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The resistance of bacterial spores to lethal agents and their extreme longevity in specific circumstances (Hastings, 1923; Wilson and Shipp, 1939; Jacotot and Virat, 1954) might suggest that spores in the main are insensitive to their natural surroundings and in consequence have an assured long life expectancy. In reality, the duration of life in aging spores is not so easily predicted, conditioned as it is both by internal, hereditary considerations and by various external, environmental factors.

Reports on spore viability in relation to aging have been limited almost entirely to pathogenic species inoculated into experimental animals, body fluids, or dried materials from such sources (Frankland, 1893; Tarozzi, 1906; Koser and McClelland, 1917; Morris, 1921; Stein, 1947; Minett, 1950; and others).

Sporogenic species of primary interest in canned foods, for the most part without health significance, have not been studied. The present paper records some observations on the influence of species and of certain environmental factors upon the long term viability of commercially significant forms.

MATERIALS AND METHODS

The following species were used: *Bacillus subtilis* (15u), American Can Company; *Bacillus brevis*, A. Bondi, Temple University; *Bacillus macerans* (7069); *Bacillus stearothermophilus* (1518), *Clostridium botulinum* (62A), and *Clostridium* spp. P.A. (3679), National Canners Association. The aerobic spores were produced on agar slopes (nutrient agar, to which was added 1 ppm of manganese) contained in large bottles. When sporulation was complete the growth was washed off with sterile distilled water, filtered through cotton and centrifuged, the water was then decanted and the washing process repeated six times. The concentrated stock suspensions thus prepared were plated and stored at 3 C until used.

Clumps were dispersed by shaking with small glass beads. Washed spores were seeded in 0.06 M phosphate buffers (mono- and dipotassium) of differing pH and distributed in screw capped Neutra-glass tubes (8 ml per tube). One-half of the tubes of each buffer sample was heated at 85 for 15 min, or 100 C for 10 min, cooled and together with the unheated samples stored in the dark at 0, 30, and 37 C, or at 30 C only. Viable spore counts were made of each buffer sample before and after prestorage heating and after varying storage periods. In each case the counts were made by heating aliquot quantities of the buffer suspension in skim milk at 85 C for 15 min followed by subcultivation of the heated suspensions in plates or tubes. The sedimented spores after storage were resuspended in the substrate by vigorous agitation of the samples in a mechanical shaker. The counting medium for the *Bacillus* species was standard glucose nutrient agar containing 0.1 per cent soluble starch. The media components throughout were derived from single batch lots to minimize nutrient variations.

The clostridia were produced in liver broth filtered through cotton and subsequently prepared as with the aerobes. Counts were made in Prickett tubes (Miller *et al.*, 1939), containing Yesair pork infusion agar with 0.1 per cent starch, plus 0.1 per cent filter-sterilized $NaHCO_3$ and sealed with 1.5 per cent agar containing BBL thioglycolate supplement (0.1 per cent sodium thioglycolate). The composition and method of preparation of Yesair pork infusion agar was that described by Wynne and Foster (1948).

Colony counts were made after 48 hr incubation at the optimum temperature of the organism. Plates and tubes were returned to the incubator and additional colonies if any, recorded

after four additional days of incubation. Preheating as used in this paper denotes prestorage heating.

RESULTS

In suspensions of *B. macerans* there was observed (figure 1) a progressive although moderate decline in viability of the spores over a storage period of 3 years at 30 C. Viability was lost more rapidly at pH 5.0 than at pH 6.0 and 8.0 and this decline was somewhat accelerated by preheating. The longevity of *B. brevis* was greatly affected by the reaction of the substrate (figure 2). At pH 8.0 most of the spores remained viable irrespective of prestorage heating. Rather rapid and almost complete destruction of the spores occurred at pH 6.0 without preliminary heating. Contrary to expectation, viability of these spores declined less rapidly at pH 6.0 with prior heat activation than at pH 5.0 with prestorage heating. This unusual behavior was confirmed with other spore crops of this strain. Spore mortality at pH 5.0 was greatly increased by prestorage heating which is

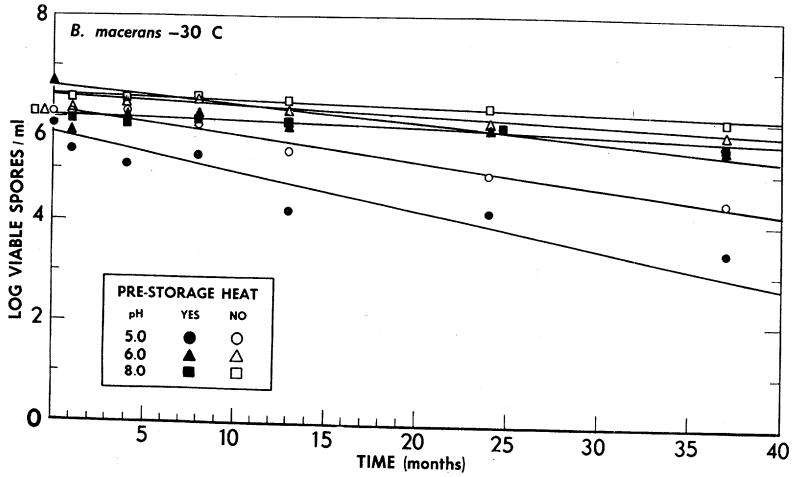

Figure 1. Effect of preheating and substrate (buffer) pH on the viability of spores of *Bacillus macerans* stored at 30 C. Preheating 85 C for 15 min.

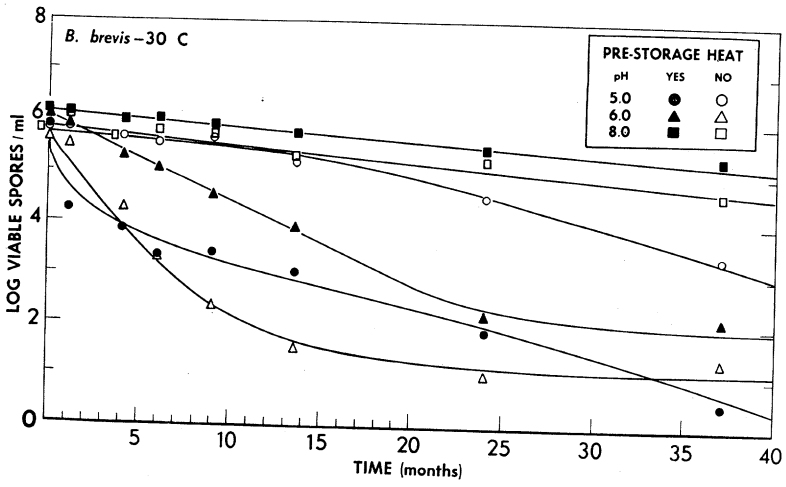

Figure 2. Effect of preheating and substrate (buffer) pH on the viability of spores of *Bacillus brevis* stored at 30 C. Preheating 85 C for 15 min.

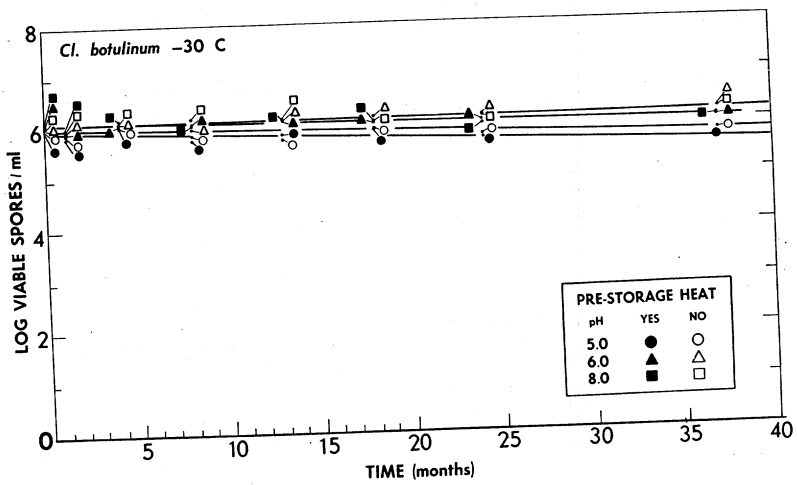

Figure 3. Effect of preheating and substrate (buffer) pH on the viability of spores of *Clostridium botulinum* stored at 30 C. Preheating 85 C for 15 min.

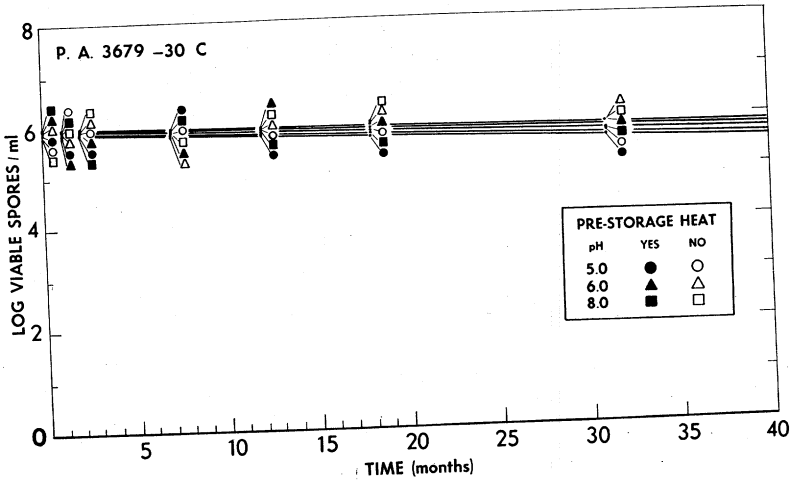

Figure 4. Effect of preheating and substrate (buffer) pH on the viability of spores of P.A. 3679 stored at 30 C. Preheating 85 C for 15 min.

in agreement with previous short-term observations of spores in distilled water (Curran and Evans, 1947).

Results obtained with spores of two anaerobes, *C. botulinum* strains 62A and P.A. 3679, are shown in figures 3 and 4. Unlike the mesophilic aerobes, the spores of both cultures retained their viability practically unchanged over a period of 3 years at 30 C. The capacity of survival of these spores was not appreciably influenced either by prestorage heating or by reaction variations within the range observed.

With two other cultures, *B. subtilis* (15u) and *B. stearothermophilus* (1518), the temperature of storage was 0, 30, and 37 C and the pH 4.0 as well as the less acid substrates previously used (figures 5 to 12).

At pH 8.0 (figure 5), spores of *B. stearothermophilus* retained their viability in storage with little change, irrespective of incubation temperature or prestorage heat shock. At pH 6.0 (figure 6), a very slow decrease in the number of viable spores occurred at 37 C. However, at pH 4.0, 37 C, following a short period of little change,

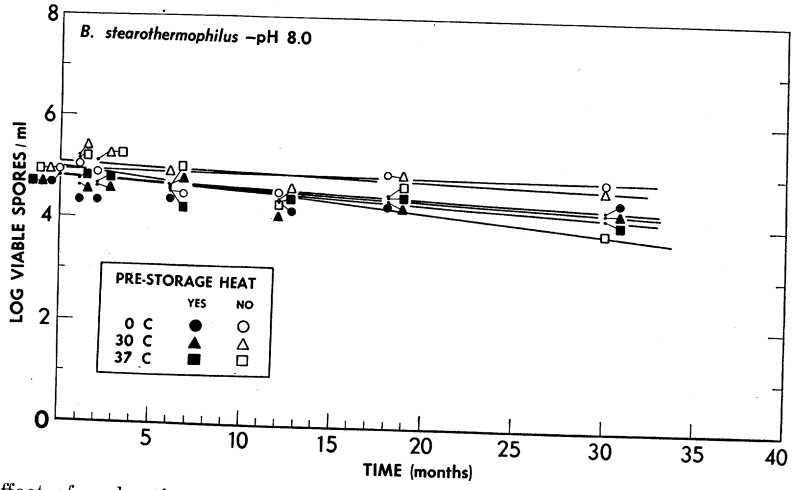

Figure 5. Effect of preheating and temperature of incubation on the viability of spores of *Bacillus stearothermophilus* in storage. Substrate (buffer) pH 8.0. Preheating 100 C for 10 min.

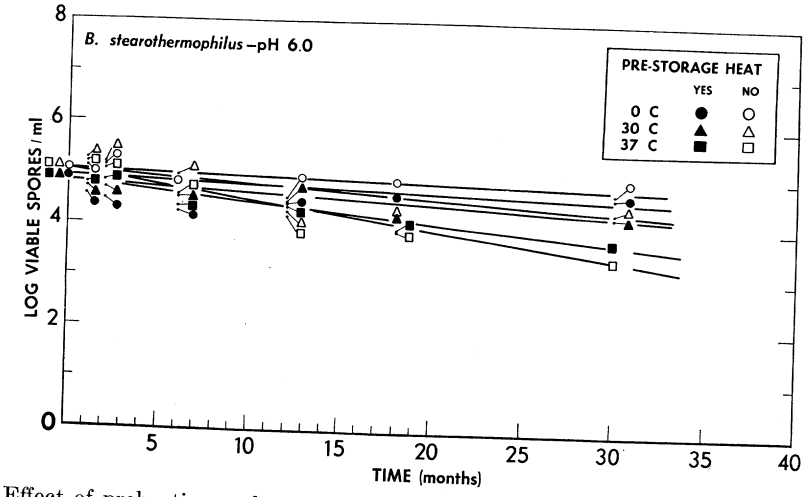

Figure 6. Effect of preheating and temperature of incubation on the viability of spores of *Bacillus sterothermophilus* in storage. Substrate (buffer) pH 6.0. Preheating 100 C for 10 min.

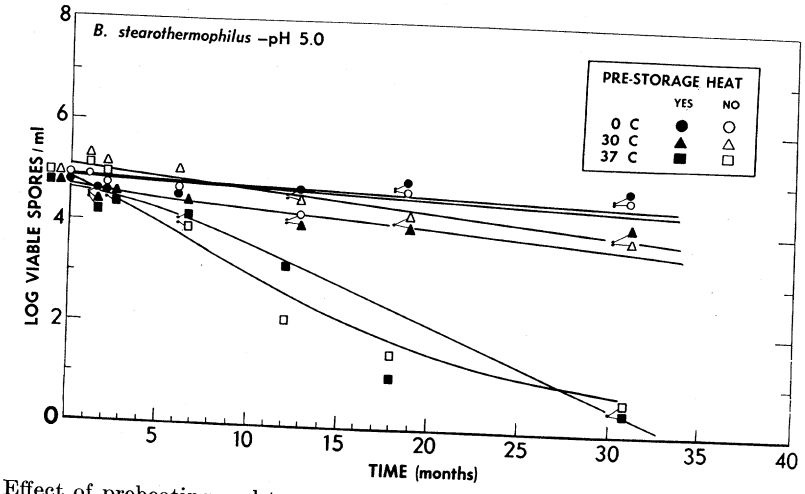

Figure 7. Effect of preheating and temperature of incubation on the viability of spores of *Bacillus stearothermophilus* in storage. Substrate (buffer) pH 5.0. Preheating 100 C for 10 min.

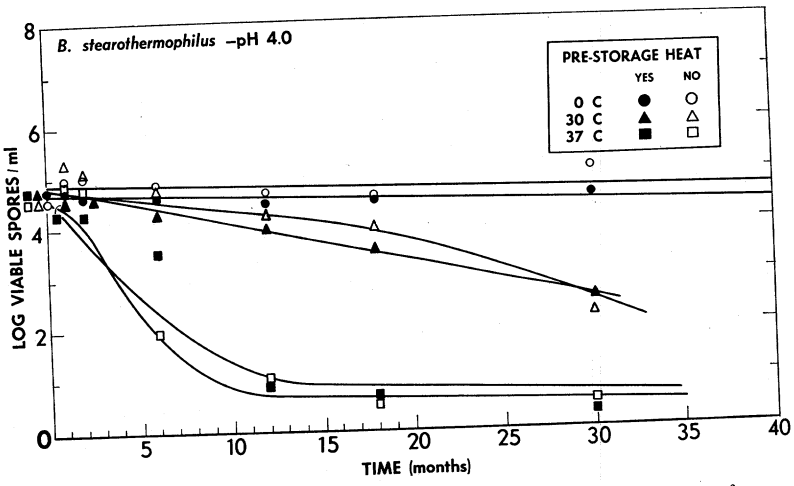

Figure 8. Effect of preheating and temperature of incubation on the viability of spores of *Bacillus stearothermophilus* in storage. Substrate (buffer) pH 4.0. Preheating 100 C for 10 min.

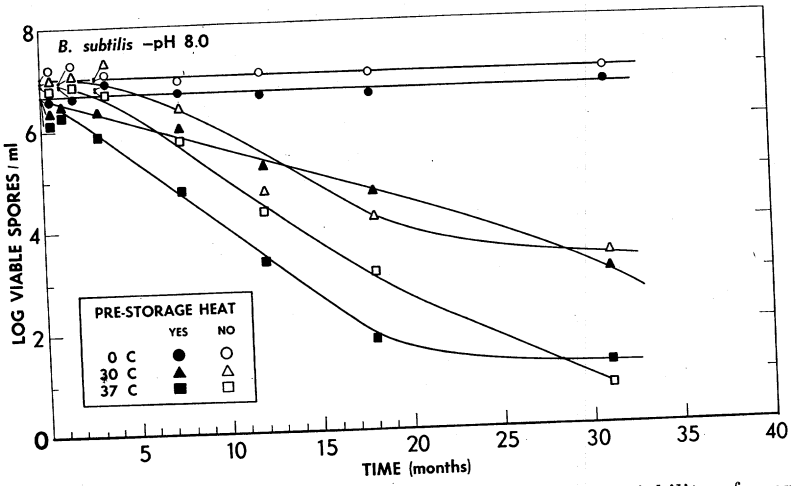

Figure 9. Effect of preheating and temperature of incubation on the viability of spores of *Bacillus subtilis* in storage. Substrate (buffer) pH 8.0. Preheating 100 C for 10 min.

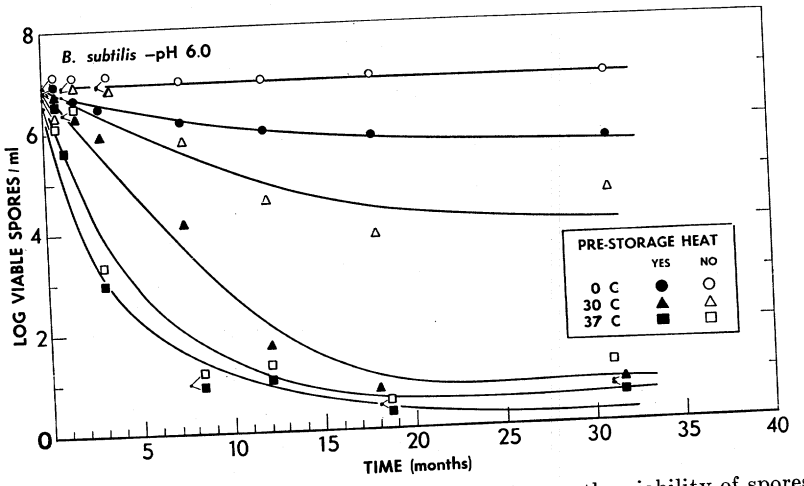

Figure 10. Effect of preheating and temperature of incubation on the viability of spores of *Bacillus subtilis* in storage. Substrate (buffer) pH 6.0. Preheating 100 C for 10 min.

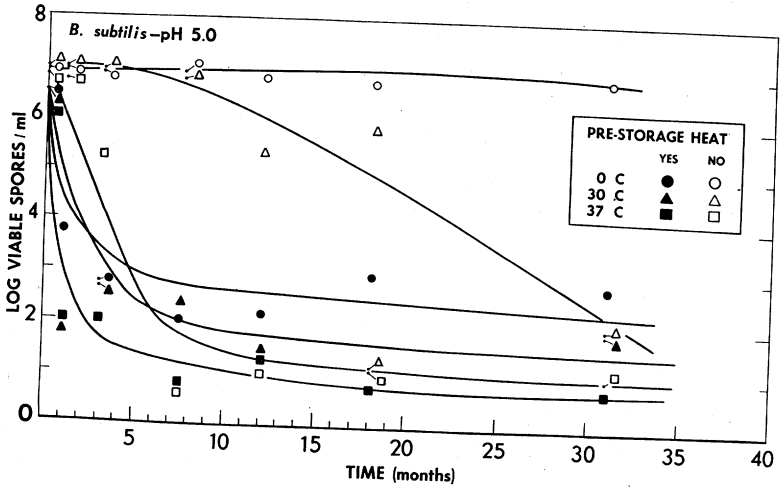

Figure 11. Effect of preheating and temperature of incubation on the viability of spores of *Bacillus subtilis* in storage. Substrate (buffer) pH 5.0. Preheating 100 C for 10 min.

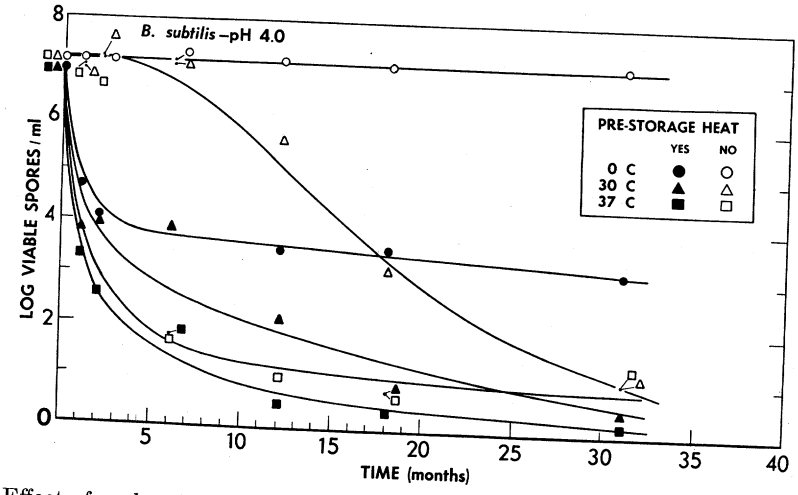

Figure 12. Effect of preheating and temperature of incubation on the viability of spores of *Bacillus subtilis* in storage. Substrate (buffer) pH 4.0. Preheating 100 C for 10 min.

viability declined quite rapidly reaching an almost stationary low level after about 12 months. At pH 5.0 (figure 7), 37 C, the results differed from the preceding chiefly in the time required to drop to the low level. That temperature was critical in the devitalization of the spores at pH 4.0 (figure 8), is indicated by comparison with the results obtained at 30 and 0 C.

Preheating of *B. subtilis* spores promoted rapid loss of viability in spores stored in acid media at 37 and 30 C (figures 10 to 12), although at 37 C the viability curve dropped sharply even in the absence of prestorage heat. Unlike the other species, there was a substantial, continuing decrease in the number of viable spores at pH 8.0 (figure 9), both at 30 and 37 C. In contrast also with other species, preheating caused a rapid decline in the viable spore population stored at 0 C, pH 4.0 (figure 12), which at pH 5.0 was less rapid.

DISCUSSION

It is apparent that spores of different species vary widely in their susceptibility to prolonged environmental influences. Preheating activates

certain enzymes (Church and Halvorson, 1955; Murty and Halvorson, 1956; Krask, 1956) tending toward, but not achieving, germination with significant loss of dipicolinic acid (Harrell 1956). When this takes place under conditions unfavorable to further development, it might be expected to weaken the spore and thus shorten its period of survival. Such an effect of preheating was observed in the aerobic spores, but not with the anaerobes. Both *C. botulinum* and P.A. 3679, however, are heat activatable, as demonstrated by Reynolds and Lichtenstein (1949). Conceivably these spores may, after activation in the absence of external nutrients, undergo prompt reversion to the inactivated condition and thus escape the enfeebling effects of continued static existence in a partially active, physiological condition.

It is known that temperatures at 37 C and below slowly activate spores if exposure is sufficiently prolonged. This was evident with the aerobes in the usual convergence of the preheated and nonpreheated viability curves after long storage at 37 C.

On traditional grounds, it might be supposed that the presence of dissolved oxygen in the storage substrate would retard or prevent incipient development of the clostridia and thus make for greater stability; however, as indicated by Wynne and Harrell (1951) and Wynne *et al.* (1952), the presence of oxygen does not necessarily inhibit beginning germination associated with loss of heat stability (labilization) and this reaction is known to occur readily in aerobic species under suitable conditions, in strictly anaerobic substrates (Roth and Lively, 1956).

It may be more than a coincidence that the cultures which grew most luxuriantly and produced spores in greatest abundance, were most vulnerable to long-term external influences, suggesting a compensatory provision of nature to insure their survival; that spores of aerobes do in fact lead a more precarious existence in nature than those of anaerobes is also suggested by the report of Koser and McClelland (1917), who recovered the spores of four pathogenic anaerobic species months after inoculation into rats, whereas spores of two aerobic species similarly inoculated and in greater numbers, were recovered only irregularly.

SUMMARY

The influence of certain factors upon the viability of bacterial spores in fluid storage was observed. In most instances prestorage heat shock caused increased loss of viability during storage. Spores of *Clostridium botulinum* 62A and of P.A. 3679, in contrast to those of aerobic species, maintained their viability almost without change over a period of 3 years. Viability of the aerobes was maintained much longer at pH 8.0 than in the acid substrates. The rate of decline in the spore populations was characteristically a function both of pH and temperature.

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